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## **Development of an in-house ELISA to detect anti-HPV16-L1 antibodies in serum and dried blood spots**

Ramya Bhatia<sup>1#</sup>, June Stewart<sup>2</sup>, Sharon Moncur<sup>2</sup>, Heather Cubie<sup>3</sup>, Kavanagh Kim<sup>4</sup>, Kevin GJ Pollock<sup>5</sup>, Camille Busby-Earle<sup>6</sup>, Alistair RW Williams<sup>7</sup>, Sarah Howie<sup>2\*</sup> and Kate Cuschieri<sup>8\*</sup>

Human Papillomavirus Research Group, Division of Pathology, University of Edinburgh, Edinburgh, United Kingdom<sup>1</sup>, Centre for Inflammation research, University of Edinburgh, Edinburgh, UK<sup>2</sup>, Global Health Academy, University of Edinburgh, Edinburgh, United Kingdom<sup>3</sup>, Department of Mathematics and Statistics, Strathclyde University, Glasgow, UK<sup>4</sup>, Health Protection Scotland, Glasgow, UK<sup>5</sup>, Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh, Edinburgh, United Kingdom<sup>6</sup>, Division of Pathology, University of Edinburgh, Edinburgh, UK<sup>7</sup>, Scottish HPV Reference Laboratory, NHS Lothian, Royal Infirmary of Edinburgh, Edinburgh, United Kingdom<sup>8</sup>.

\* Contributed equally to the work

# Address correspondence to Ramya Bhatia, ramya.bhatia@ed.ac.uk

**Running Title: Systemic and cervical antibody responses to HPV16**

# Abstract

Measuring anti-HPV antibody levels is important for surveillance of the immunological response to both natural infection and vaccination. Here, an ELISA test for measurement of HPV-16L1 antibodies was developed and validated in sera and dried blood spots.

An in-house ELISA was developed for measuring anti-HPV-16L1 IgA and IgG levels. The assay was standardized against WHO international standard serum and validated on serum, dried blood spots and cervical liquid based cytology samples from women attending colposcopy clinics in Scotland. Antibody avidity index was also measured in serum samples.

The average HPV 16-L1 specific IgG and IgA levels measured in sera, in women attending a routine colposcopy service were 7.3 units/ml and 8.1 units/ml respectively. Significant correlations between serum and dried blood spot eluates for both IgG and IgA were observed indicating that the latter serve as a credible proxy for antibody levels. Average IgG Avidity Index was 35% (95% CI 25%-45%) suggesting previous, historical challenge with natural infection. This ELISA has potential for use in epidemiological and field studies of antibody prevalence and if coupled with avidity measurement may be of use in individual case monitoring of vaccine responses and failures.

**Keywords: HPV16, Antibody response, Avidity, Dried Blood spots**

# Introduction

Human Papillomavirus (HPV) infections are very common in the general population with an estimated 10-15% prevalence in women of cervical screening age (Crow, 2012). While most HPV infections are cleared naturally within 2 years, persistent infection with one of the “high-risk” HPVs (HR-HPV) is associated with development of cervical cancer. Two high-risk types, 16 and 18, are associated with over 70% of cervical cancers (Crow, 2012), which has informed prophylactic vaccine design and implementation. In addition, HR-HPV infection is strongly associated with development of vaginal, vulval, penile, anal and oropharyngeal cancers (Schiffman et al., 2016).

As only a minority of those infected with HPV develop disease the host immune response is clearly important in the regulation and trajectory of the infection. This is also evidenced by the fact that immunocompromised individuals are at a greater risk of HPV-associated clinical morbidity (Reusser et al., 2015). Antibodies are important for protective immunity against HPV infection and neutralising antibodies against the major capsid protein L1 of the virus are efficacious against infection with 16/18, which is the rationale behind vaccine design. Currently three prophylactic vaccines are licensed for use – the bivalent vaccine which confers protection against types 16 and 18, the quadrivalent vaccine which protects against 16, 18, 6 and 11 and the nonavalent vaccine which protects against HPV 6, 11, 16, 18, 31, 33, 45, 52 and 58. The rate of seroconversion associated with the licensed vaccines is high, with reports of >99% seroconversion in both immunocompetent males and females (Paavonen et al., 2007; Van Damme et al., 2015; Villa et al., 2006; Viscidi et al., 2004). Comparatively seroconversion as a consequence of natural infection is low with rates of around 50-70% in women (Carter et al., 2000) and 4-36% in men (Edelstein et al., 2011), with the average time to seroconversion 8-9 months after HPV infection (Carter et al., 2000). In addition,

concentrations of anti-HPV16L1 serum antibody associated with natural infection are significantly lower than levels generated as a consequence of vaccination (Carter et al., 2000). The lower titres of antibodies generated as a consequence of natural infection, when compared to those generated by the vaccine, are due to the non-lytic nature of the virus, the lack of viraemia and the fact that the epithelia, (the obligate target cells for natural infection) are relative sparse with respect to immune effector cells. In comparison as the vaccine is delivered by intra-muscular injection(s) more immediate and comprehensive exposure to immune effector cells generates high titres of antibodies.

HPV serology is an essential tool which can provide key insight into the natural history of HPV infection particularly given that molecular detection of HPV using nucleic acid amplification tests cannot determine previous infection. Furthermore, serological tools provide valuable information on the longevity and magnitude of antibody responses to vaccination which, through linkage to clinical outcomes, are essential in informing vaccine regimens and indications, as well as vaccine failures. Avidity testing can additionally inform the quality of antibody response. However, compared to molecular HPV assays which number over 200 (Poljak et al., 2016) there are relatively few HPV serology tests, particularly non-proprietary ones. Most serology tests are in-house and not standardised against the WHO International standard sera (Bissett et al., 2012). Another challenge in conducting sero-epidemiology is the practicality of obtaining and processing venous blood, particularly in the context of large population based studies. Dried blood spots offer clear operational advantages in terms of capture, storage and transport, although to our knowledge only two studies have determined the feasibility of this approach (Louie et al., 2018; Waterboer et al., 2012).

In light of this, the objectives of the present work were to develop an in house ELISA for the measurement of anti-HPV 16-L1 antibodies and perform avidity measurement. The ELISA

was calibrated to the WHO international serum standard. Application of the assay in a prospective colposcopy population allowed antibody titres in serum and dried blood spots to be compared.

## **Materials and Methods**

### **Governance**

Ethical approval was obtained for prospective sample collection from Scotland A Research Ethics Committee (REF 12/SS/0034).

### **Colposcopy cohort from prospective collection- venous blood and cervical liquid based cytology (LBC) samples**

The British Association for Cytopathology (BAC) reporting guidelines and the cervical intraepithelial neoplasia (CIN) nomenclature were used to classify cytological findings and histological outcomes, respectively (Smith and Patnick, 2013). Management of women with abnormal cytological results was performed according to guidelines and algorithms associated with the United Kingdom National Health Service Cervical Screening Programme (NHS CSP), modified for use in the Scottish context (Hirschowitz, 2012; Smith and Patnick, 2013). Unvaccinated women attending the NHS Lothian Colposcopy clinics after a diagnosis of moderate or severe cytological abnormality were recruited to the study. The median age was 29 years (21-59 years). Informed consent was obtained from participants for provision of a blood and LBC sample at the time of colposcopy visit. Biopsies were performed as routinely indicated, and study results did not influence clinical management. A total of 96 serum samples were collected of which 95 had matching LBC samples (Figure 1).

### **Clinical sample collection and processing**

#### **Blood**

Venous blood was collected and serum and blood spots were prepared. Blood spots were prepared using two 75 µl spots on Guthrie spot filter papers. The spots were air dried and stored at 4°C for 24hrs following which blood was eluted using 800µl PBS/0.05% Tween. Serum was separated from the remaining blood by centrifugation at 12000g.

#### Cervical LBC samples

Cervical liquid based cytology samples derived from the clinic were collected into 20ml of Preservcyt. Samples were passed through a blunt needle three times to make a uniform cell suspension, aliquotted and stored at -20°C.

#### **HPV testing of LBC samples**

Genotyping was performed on the LBC samples using the Optiplex HPV Genotyping Kit (Diamex GmbH) (Kavanagh et al., 2013) which genotypes 18 high-risk or putatively high-risk types and 6 low-risk types according to the current IARC classification.

#### **ELISAs for anti-HPV16 IgG and IgA antibodies in sera and blood spots**

Human International Standard (IS) serum against HPV16 (WHO International Standard 05-134 HPV 16 antibodies NIBSC code: 05/134) was obtained from The National Institute for Biological Standards and Control (NIBSC, South Mimms, UK) and reconstituted to a concentration of 10 International Units (IU) of HPV16 L1 specific antibody/ml according to instructions (Ferguson et al., 2011). To detect high affinity antibodies which reflect a strong immune response, 2-fold dilution(s) from 1 in 1000 to 1 in 64,000 of the IS serum were made and used to create a standard curve (Fig 2a). The standard curves were tested 8 times and in duplicate on each subsequent plate. All serum samples were tested in duplicate at 1 in 2000 dilutions.

For consistency between the amount of total protein in serum and blood spot eluate dilutions, we calculated the lowest amount of protein in serum as 3 µg/ml at a 1 in 2000 dilution. Therefore, blood spot samples were tested at a protein concentration of 3 µg/well. It should

be noted that protein in the blood spot eluates also contained protein from all blood cells as well as plasma.

Successful IgG and IgA binding with the IS sera for HPV16L1 protein (AbCam, Cambridge, UK) was obtained when the antigen was used at 50ng/well (500ng/ml) (data not shown,) so this concentration was used subsequently for all samples. HPV16L1 protein was diluted in 100  $\mu$ l PBS and coated onto ELISA grade 96 well NUNC MaxiSorp<sup>®</sup> plates (eBiosciences, Hatfield UK) by overnight incubation at 4°C. The wells of the plate were decanted and blotted on tissue paper, washed x5 with PBS containing 0.1% Tween20 (wash buffer). ELISA buffer (100  $\mu$ l PBS containing 1% BSA) was added to each well to block non-specific binding. IS serum against HPV16L1 protein was pre-absorbed against BSA by diluting to 1 in 200 with ELISA buffer, incubating overnight at 4 °C followed by centrifugation at 20,000g for 15 min in a benchtop microfuge to remove any complexes. The IS serum was then diluted in ELISA buffer, added to wells and incubated for 1h at room temperature. Plates were washed as above and 100  $\mu$ l biotinylated-anti-human IgG (1 in 100,000 dilution), or biotinylated-anti-human IgA (1 in 10,000 dilution) both from Vector Laboratories Ltd (Peterborough, UK), were added to the wells and incubated for 1h at room temperature. Plates were again washed as above. A 100  $\mu$ l volume of streptavidin-horseradish peroxidase complexes (Vector Laboratories Ltd (Peterborough, UK)) was added to each well and incubated for 1h at room temperature before plates were again washed as above. Subsequently, a 100  $\mu$ l volume of TMB (SIGMA (Poole, UK) reagent was then added to each well and plates incubated at 37°C for 30 minutes before the colour reaction was stopped with 1N sulphuric acid. An automated dual filter plate reader was used to measure absorbance at 450nm/630nm.

#### **Antibody avidity measurement**



To determine the avidity index of IgG, we utilised the guanidine hydrochloride method (Inouye et al., 1984) that has been used previously in studies of anti-HIV (Chawla et al., 2007) and anti-HPV IgG antibodies (Dauner et al., 2012) in human serum. Initial experiments established that 2M guanidine hydrochloride (GuHCL) treatment gave optimal results in the ELISA. ELISA was performed as described above except GuHCL treatment was performed for 15 minutes before addition of biotinylated anti-human IgG. Avidity index was measured as the percentage of IgG (IU/ml) with GuHCL treatment divided by IgG (IU/ml) without treatment. GuHCL has been reported as a chemical that disrupts the strength of binding between antibody and antigen and in particular does this without disrupting the VLP structure of HPV 16-L1. Thus GuHCL was used for the present study

### **Statistics**

Analysis was performed using Graphpad Prism™. Data were tested to determine whether there was correlation between IgG and IgA in the same samples and between different types of samples. A p-value < 0.05 was considered to be statistically significant.

### **Results**

#### **Clinical and viral annotation of samples**

Of the 96 women in the study cohort with moderate or severe cytological abnormalities, 5 (5.2%) had a biopsy which was reported as normal, 1 woman (1.05%) had CIN1, 40 (41.67%) had CIN2, 43 (45.2%) had CIN3, 2 (2.1%) had high grade cervical glandular intraepithelial neoplasia (CGIN), 1 (1.05%) had small cell cervical carcinoma (SCCC) and 4 were missing or not definitive (Figure 1).

HPV genotyping was performed on LBC samples from 95 women (one woman had no associated LBC sample collected). A total of 92 (95.7%) women tested positive for any HPV, 52 had mono-infections and the remainder of HPV positive women had  $\geq 2$  infections. HPV16/18 were the most common types (48/91, 52%). In total 72 (75.7%) women were

currently infected with HPV 16 or a related virus in the alpha species group 9 and 20 women were infected with other HR-HPV types (Figure 1).

#### **Level of anti-HPV16-L1 antibodies in sera**

Anti-HPV16-L1 antibody levels as measure by ELISA in sera from the 96 women were measured against standard curves of the IS serum (Figure 2a&b). The average HPV 16-L1 specific IgG and IgA levels in the colposcopy population was 7.3 units/ml and 8.14 units/ml respectively. There was significant correlation between the levels of IgG and IgA anti-HPV16-L1 antibodies in the sera (two-tailed Spearman  $r=0.5094$ ,  $p<0.0001$ ) (Figure 2c) of the women. There was no significant difference between anti-HPV16-L1 IgG or IgA levels according to underlying histology (Kruskal Wallis test; no disease IgG versus CIN2+ IgG,  $p=0.7531$ ; no disease IgA versus CIN2+ IgA  $p=0.9905$ ).

#### **Level of anti-HPV16-L1 antibodies in blood spot eluates**

All blood spot eluates were tested for total protein concentration and the mean  $\pm$  standard error of mean (s.e.m) concentration was  $1652 \pm 41.78$   $\mu\text{g/ml}$ . Data for blood spots are given in units of anti-HPV16-L1 specific antibody (IU)/100 mg protein (Figure 3a) calculated against standard curves of the IS serum.

Average IgG level in blood spots in the colposcopy population was 3.94 units/ml and the average IgA level was 12.71 units/ml. There was a significant correlation (two-tailed Spearman  $r=0.4900$ ,  $p<0.0001$ ) between IgG and IgA antiHPV16L1 antibodies in the blood spot eluates (Figure 3b). There were also significant correlations between serum and dried blood spot eluates for the levels of IgG, two-tailed Spearman  $r=0.2815$ ,  $p=0.0063$  (Figure 3c) and IgA, two-tailed Spearman  $r=0.5485$ ,  $p<0.0001$  (Figure 3d).

#### **Anti-HPV16-L1 antibody levels according to HPV infection**

To determine whether serum antibody levels reflected current infection, all serum samples from women with HPV infection were stratified according to a) having a HPV16 or a

phylogenetically related type which resided in the alpha species group 9 or b) having an other HPV type, outside the alpha 9 group. Figure 4 shows that, although there was a range in levels of anti-HPV16L1 antibody, sera from women currently infected with at least one alpha 9 type (the majority of whom were HPV 16 positive (n=48/72 alpha 9 positive women), showed significantly higher values of both IgG (Figure 4a) and IgA (Figure 4b) anti-HPV16L1 antibodies compared to those infected with other HPV types.

#### **Avidity index measurement in serum**

Antibody avidity (AI) in sera from 34 women who had IgG values for HPV16 of 75% or above (relative to the International Standard serum) (Figure 5) was assessed. The average avidity index in the sample set was 34.86 (95% CI- 24.84- 44.88) and of the 34 sera, 33 showed a significant decrease in antibody levels following GuHCL treatment; the mean  $\pm$  s.e.m % decrease was  $65.14 \pm 4.929\%$ .

#### **Discussion**

Efforts at detection of HPV antibodies as a tool to monitor and assess vaccine efficacy have increased significantly in recent years. Several assays, designed to measure HPV antibody responses in serum are available such as GST- L1 multiplex test (Waterboer et al., 2005), cLIA (Wentzensen et al., 2011) and in house ELISAs (Mesher et al., 2016). However, there is a lack of harmonization and standardization of these tests which makes it challenging to compare data from different studies.

In the present study a reproducible in-house ELISA was developed using widely available reagents which can robustly detect HPV 16 IgG and IgA in serum and blood spots. The assay has been calibrated to the international HPV16 serum standard developed by WHO (Pagliusi et al., 2006). The ability to use dried blood spots has implications for both low resource and primary care settings as they only require taking a droplet of blood and can be transported to

the lab at ambient temperatures. A correlation between the levels of IgG and IgA was found in serum, blood spot and LBC samples. A strong correlation between anti-HPV16L1 IgG and IgA in serum and blood spots was also observed. The present study thus independently validates the studies/observations of Waterboer et al (2012), and more recently Louie et al (2018) and demonstrates the feasibility of using dried blood spots for surveillance purposes. In addition, the present work we reference to an international standard (which Waterboer et al did not incorporate) showed that the ELISA can measure both IgA and IgG effectively.

Another objective of this study was to use the ELISA to investigate the avidity of IgG antibodies which may provide insight into vaccine efficacy during the longitudinal monitoring of vaccinated cohorts.

Avidity is the measurement of the strength of interaction between the specific antibody and the antigen it recognises. During the course of an immune response, antibodies of increasing avidity for antigen are produced and selected by somatic mutation of the immunoglobulin genes in antibody producing B lymphocytes. Generally, avidity of IgG antibodies increases over the 4-6 months following a cleared infection and declines thereafter. Thus a high avidity measurement indicates a recent or persistent infection. Natural infection with HPV has been associated with low avidity antibodies as demonstrated recently by Louie et al 2018. Furthermore, the avidity index (AI) observed in the present study is similar to that observed in the study of Scherpenisse et al (2013) who reported around 35% AI associated with natural infection and that the AI of vaccine induced antibodies was around 3 times higher. AI is therefore, to an extent a proxy of immunisation-induced antibodies and notably reports that AI were similar between a 2-dose and 3-dose regimen supported the rationale for 2-dose regimens (Sankaranarayanan et al., 2016).

In conclusion, in this study, a standardised ELISA test developed for anti-HPV16L1 antibodies was validated against the WHO international positive serum standard for HPV16. This assay was amenable to both venous blood and dried blood spots. While the sample size used for this study is small, this technique has promise for widespread use in epidemiological and field studies of antibody prevalence and coupled with the avidity measurement may be of use in individual cases for monitoring vaccine responses such as failures. The existence of serological assays which are calibrated to the same standards will also facilitate cross-study comparison in the future.

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## Figure legends

**Figure 1. Underlying pathology of the cervical sample set.** Details of samples used within the study. CIN- Cervical Intraepithelial Neoplasia, CGIN- Cervical Glandular Intraepithelial Neoplasia, SCCC – Small Cell Cervical Cancer, HR-HPV – high –risk Human Papilloma virus.

**Figure 2. Measurement of Anti HPV 16 L1 antibodies in sera by ELISA**

(A) Linear regression of ELISA standard curves. Mean (solid lines) and 95% confidence intervals (dashed lines); red lines = IgG; black lines = IgA. Plates coated with 50ng/well HPV16L1, n=8;  $r^2$  for IgG=0.8872;  $r^2$  for IgA = 0.9371. OD= optical density. (B) Levels of IgG and IgA in serum samples calculated against the IS serum standard. Bars show mean  $\pm$  95% confidence interval, n=94. (C) Correlation between levels of anti-HPV16 L1 IgG and IgA antibody in sera; n=94; two-tailed Spearman  $r=0.5248$ ,  $p<0.0001$ .

**Figure 3. Measurement of Anti HPV16 L1 antibodies in eluates from dried blood spots.**

(A) IgG and IgA antibody, bars show mean + 95% confidence interval, n=94. (B) Correlation between levels of anti-HPV16 L1 IgG and IgA antibody in blood spots; n=94; two-tailed Spearman  $r=0.4900$ ,  $p<0.0001$  (C) Correlation between levels of anti-HPV16 L1 IgG antibody in serum and blood spots; n=95; two-tailed Spearman  $r=0.2815$ ,  $p=0.0063$ . (D) Correlation between levels of anti-HPV16 L1 IgA antibody in serum and blood spots; n=95; two-tailed Spearman  $r=0.5485$ ,  $p<0.0001$ .

**Figure 4. Measurement of Anti HPV16 L1 antibodies in sera from women with and without infection with HPV 16 (or related types)** (A) IgG and (B) IgA levels in serum from

women with HPV 16 or a virus that resides in the alpha 9 species group, where HPV 16 is located (n=72). This is compared to sera from women with unrelated virus types (n=20). Bars show 95% confidence interval(s), Mann Whitney Test



**Figure 5. Measurement of IgG avidity in sera following treatment with guanidine hydrochloride (GuHCl).** HPV16L1 binding antibodies in 34 sera with IgG levels >75% of the IS serum were compared before and after GuHCl treatment. (A) 33/34 sera showed a reduction in avidity after treatment;  $p < 0.001$ , Wilcoxon signed rank test. (B) Avidity index of antibodies in sera; bars show mean  $\pm$  95% confidence interval.